UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

FEB 24 1992

OFFICE OF
PESTICIDES AND TOXIC
SUBSTANCES

MEMORANDUM

SUBJECT: Registrant Request to Waive Tier III Aquatic Plant Growth Testing for Tebuthiuron. DP BARCODE: D160688

FROM: Douglas J. Urban, Acting Chief
Ecological Effects Branch
Environmental Fate and Effects Division (H7507C)

TO: Louis Rossi, Chief
Reregistration Branch
Special Review and Reregistraton (H7508W)

On October 24, 1990 representatives of DowElanco met with members of the Ecological Effects Branch (EEB) to discuss nontarget plant data requirements for tebuthiuron. At this meeting the registrant expressed an interest in having the Tier III requirement waived. They were requested to formally submit a waiver request to the Agency outlining their rational for the waiver. The waiver request, dated January 9, 1990, has been provided along with documents DowElanco feels support conclusions that Tier III aquatic plant testing is not justified. EEB has reevaluated the aquatic nontarget plant data for tebuthiuron and considered the points made by DowElanco in their submission regarding test procedures, statistical analysis of nontarget plant data, monitoring studies, and current registered application rates for tebuthiuron.

In the studies reviewed for Anabaena flos-aquae, Skeletonema costatum, and Navicula pelliculosa (MRID's 41080401, 41080402, and 41080403) it was noted that aluminum foil was placed on top of the flasks to prevent contamination. This fact did not result in a lower evaluation of the studies. All three were classified as core.

In the toxicity study reviewed for Lemna gibba (MRID 41080404) it was noted that three plants per replicate were used rather than 5 plants per replicate. As pointed out by DowElanco, this was discussed with EEB by Lily Research Laboratories prior to the start of the study. This modification did not result in a lowering of the classification. The study was classified as core.

DowElanco has calculated EC50 values for the 5 aquatic plant indicator species using growth rates. The resulting EC50 values are higher than those calculated by EEB, which as a matter of branch policy, utilizes standing crop (biomass) values or frond counts (L. gibba). In addition, EEB uses probit analysis to determine the EC50

values for aquatic plants. DowElanco does not feel this is appropriate. Environmental Fate and Effects Division (EFED) statisticians are currently evaluating the methods employed by EEB to analyze nontarget plant data. The reference provided by DowElanco will be utilized, along with other published documents, to determine the most suitable statistical methods. Following this review by EFED, EC50 values will be recalculated.

These differences in methods of data analyses and selection of endpoints have resulted in EC50 values that are dissimilar (DowElanco EC50 values are higher). The following is a comparison of the two sets of values:

EC50 Values Calculated by DowElanco and EEB

	DowElanco	EEB
Lemna gibba	0.235 ppm	0.135 ppm
Selenastrum capricornutum	0.305 ppm	0.049 ppm
Anabaena flos-aquae	30.900 ppm	4.060 ppm
Skeletonema costatum	0.101 ppm	0.067 ppm
Navicula pelliculosa	0.213 ppm	0.081 ppm

Tebuthiuron is registered for the control of brush and woody plants at a maximum application rate of 6 lb ai/A in noncrop areas such as: railroad and utility rights-of-way, wildlife plantings, industrial sites, pipelines, fencerows, firebreaks, ditchbanks, and along highways. A 4 lb ai/A rate is registered for use on rangeland. Current EEB policy for a worst case scenario is to use the estimated runoff from 10 acres treated at the highest registered rate flowing into a 1 acre pond 6 inches deep. (Note that in the original EEB review dated June 12, 1990, the EEC was incorrectly calculated based on a one acre pond 6 feet deep). Using the 6 inch scenario, application at the 6 lb ai/A could result in a water concentration of 2.21 ppm (6 lb ai/A x 10 A x 5% runoff x 735 ppb = 2.21 ppm). This value exceeds the DowElanco EC50 values for 4 of the 5 indicator species (L. gibba, S. capricornutum, S. costatum, and N. pelliculosa). The EEC also exceeds the EC50 values for the same 4 species calculated by EEB. Tier III testing is required if the EC50 of one species is exceeded by the EEC. In this case, Tier III testing would be triggered by values obtained by both DowElanco and EEB.

Assuming that tebuthiuron will only be applied on a spot basis at the 6 lb ai/A rate, EEB has also calculated the EEC at the 4 lb ai/A rate. Relying on this scenario, the EEC could be 1.47 ppm (4 lb ai/A x 10 A x 5% runoff x 735 ppm = 1.47 ppm). This value exceeds the EC50 values of the same 4 species calculated by DowElanco and EEB. Again, Tier III testing would be required for aquatic species.

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Monitoring data reviewed by Environmental Fate and Ground Water Branch (EFGWB) and found acceptable were considered in EEB's previous review to better estimate the aquatic plant hazard from tebuthiuron. EEB used data from Oklahoma as a worst case scenario. DowElanco in their submission felt that the Oklahoma site was an outlier. The following summary lists the values that were reported along with the extrapolated values:

Highest Observed Tebuthiuron Concentrations in Ponds and Extrapolated Values

Texas	Idaho	Oklahoma	Arizona
28.9 A @ 2 lb ai/A	98 A @ 1 lb ai/A	11 A @ 2 lb ai/A	168 A @ 3 lb ai/A
0.07 ppm	0.002 ppm	0.18 ppm	0.05 ppm
2X = 0.14 ppm	4X = 0.008 ppm	2X = 0.36 ppm	1.3X = 0.067 ppm
3X = 0.21 ppm	6X = 0.012 ppm	3X = 0.54 ppm	2X = 0.1 ppm

In the previous EEB review, data from the Oklahoma site were extrapolated to a 6 lb ai/A application rate resulting in a value of 0.54 ppm. This value exceeded the EC50 values for 4 of the 5 indicator species (L. gibba, S. capricornutum, S. costatum, and N. pelliculosa), using the DowElanco and EEB calculated values. Estimating the concentration based on a 4 lb ai/A rate results in an extrapolated value of 0.36 ppm. This concentration also exceeds the EC50 values for the same 4 indicator species using the DowElanco and EEB calculated values. Extrapolating from the Texas data, both the 4 and 6 lb ai/A rate (EEC's 0.14 and 0.21 ppm, respectively) exceed the DowElanco EC50 for S. costatum (0.101 ppm) and the EEB EC50 values for L. gibba, S. capricornutum, S. costatum, and N. pelliculosa (0.135, 0.049, 0.067, and 0.081 ppm, respectively). Based on these extrapolated values from Texas and Oklahoma, Tier III aquatic plant testing is triggered.

Since the monitoring studies were evaluated by EFGWB and found acceptable, EEB does not feel it is unjustified in utilizing the values obtained and extrapolating to higher application rates. However, since the rate was less than the maximum allowed for rangeland and the EEC's had to be extrapolated, it may be prudent to conduct a monitoring study at the recommended rate for rangeland. This new study could provide data that would either substantiate or refute the EEB extrapolation. In addition, this would allow time for the evaluation of EEB's statistical methods and complete the proposed workshop and guidance document for Tier III aquatic nontarget plant testing. Should this new monitoring study trigger higher tier testing, the appropriate guidance would be available to the registrant. Testing at the Tier III level should be waived until the monitoring study is complete and the data evaluated. The protocol for the monitoring study should be submitted to EEB for review prior to initiation of the study.

DowElanco
9002 Purdue Road
Indianapolis, IN 46268-1189

January 9, 1991

DowElanco

Ms. Carol Peterson
Document Processing Desk RS-0054
Office of Pesticide Programs H7504C
U.S. Environmental Protection Agency
401 M Street
Washington D.C. 20460

Dear Ms. Peterson:

RE: TEBUTHIURON REGISTRATION STANDARD
EPA REG. NO. 62719-109
FOLLOW-UP TO OCTOBER 24, 1990, MEETING ON TIER III TESTING REQUIREMENTS

On October 24, 1990, a meeting was held with representatives of DowElanco and personnel from the Ecological Effects Branch for the purpose of reviewing the status of requirements for Tier III testing of tebuthiuron. The meeting was intended to check for accuracy of understanding regarding the study reviews, gain a current understanding of EPA testing and policy positions, provide a clear and accurate picture on how tebuthiuron is used, and to share what exposure risks may be expected based on actual commercial use. The focus for this discussion was on Tier III aquatic issues but the information had application to terrestrial risks as well. After review of information presented at this meeting, the representatives from EPA suggested that DowElanco request in writing a re-evaluation of the risk assessment previously performed which first triggered the proposal for Tier III environmental studies. DowElanco is documenting the background information shared at that meeting with this submission and is formally requesting re-evaluation as to the need for Tier III environmental studies. The following is detailed here:

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Product Use, Site, and Application Information

Product Forms

Formulation	Primary Uses	Application Equipment
80% Wettable Powder	•Broadcast for Total Vegetation Control •Banded for Brush Control	•Ground Operated Spray Rigs
5% Granule	•Broadcast for Total Vegetation Control	•Ground Operated Granular Spreaders
6% Granule (Tebuthiuron 2% Trifluralin 4%)	•Broadcast for Total Vegetation Control	•Ground Operated Granular Spreaders
20% and 40% Pellets	•Broadcast or Spot Applied for Brush Control	•Ground Or Aerial Application

Product Uses and Rates of Application

The maximum label use rate of tebuthiuron is 6 lb a.i./A. However, this rate is generally used on a limited basis to provide total vegetation control to small target areas such as around buildings, tank farms, railroad yards, power stations, etc. A typical use rate for larger areas (rangeland) is 0.5 to 2 lb a.i./A. The maximum rate allowed for use on rangeland is 2 lb a.i./A in areas receiving less than 20 inches of rainfall and 4 lb a.i./A in areas receiving 20 inches or more rainfall per year.

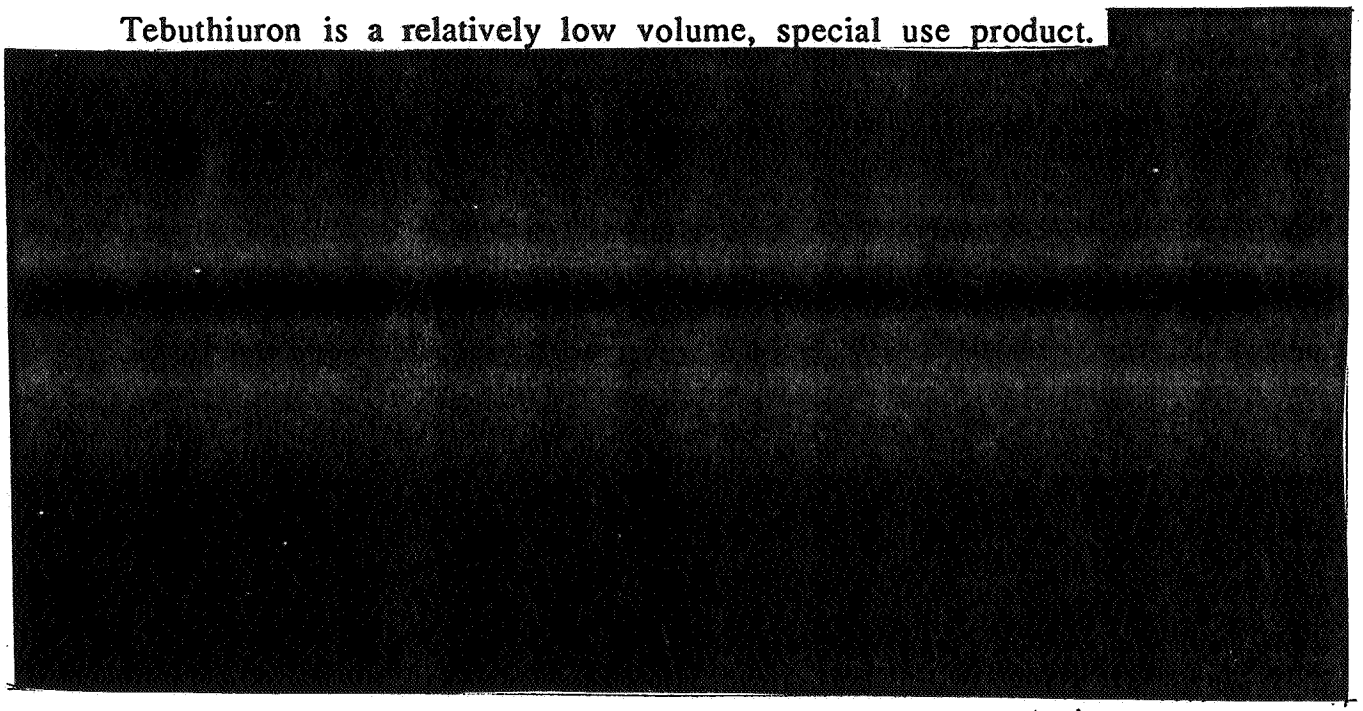
The highest levels of these rate ranges for use on rangeland would only be use in restricted areas to control localized growths of difficult to control brush species. Larger areas of rangeland would receive the lowest feasible application rate to minimize injury to the desirable grasses and to keep costs to a minimum. As a point of reference, the USDA - Forest Service Environmental Impact Assessment for the Intermountain Region (including North Dakota, South Dakota, Nebraska, Kansas, New Mexico, Colorado, Wyoming, Montana, Idaho, Utah, Arizona, and Nevada) reported typical application rates for tebuthiuron as follows:

Tebuthiuron Use	Typical Rate lb. a.i./A
Rangeland	0.5
Forestland	0.5
Facilities	4.0
Rights-Of-Way	2.0
Recreation/Administrative	2.0
Riparian	1.5

The formulation used on rangeland is the 20% pellets. The pellet is cylindrical (1/8" diameter, 3/16" long) and has a bulk density of 55 to 60 lb/cu. ft. Therefore, drift from the intended application sites is very minimal, whether from ground or aerial applications. The distribution of individual 20% pellets given an application rate of 1 lb. a.i./A would be about one pellet per square foot. Individual plant treatments on rangeland by hand application would be used on brush stands that are less then 100 to 200 plants per acre. On more dense stands, broadcast applications would be made.

Market Size

Tebuthiuron is a relatively low volume, special use product.



COMMERCIAL/FINANCIAL INFORMATION IS NOT INCLUDED

Determination of Expected Environmental Exposure From Broadcast Applications of Tebuthiuron - Response to EPA Review

In estimating the expected environmental exposure of tebuthiuron in water, the Agency referred to data obtained from a field residue monitoring study conducted in Marietta, Oklahoma, (MRID 406400-03). In this study, tebuthiuron was applied to an 11 acre watershed at an application rate of 2 lbs a.i./A. Analysis of water in a catchment pond receiving runoff from the watershed showed a tebuthiuron concentration of 0.18 ppm. The Agency assumed that if tebuthiuron was applied at the maximum use rate of 6 lbs a.i./A, the expected exposure concentration in the catchment pond would be three times the concentration reported in the Marietta, Oklahoma study or 0.54 ppm.

DowElanco believes that this value is not derived from a reasonable worst-case assessment scenario. First, an unusually large rainfall occurred (i.e., 7 inches within a 24 hour time period) coupled with an unusually high yearly rainfall (i.e., 44 + inches) after application. A 7.1-inch rainfall occurs within a 24 hour time period once in every 25 years in this part of Oklahoma. (See Table 1) Second, the catchment pond volume in the Oklahoma study was only 1.5 acre feet, compared with a 6 acre-foot pond typically used by the Agency for worst-case calculations. Third, although the maximum label use rate of tebuthiuron is 6 lbs. a.i./acre, this rate is not registered for broadcast treatment on rangeland. The maximum use rate would be 4 lb a.i./A but as pointed out earlier, the normal rate would be between 0.5 to 2 lb a.i./A. The highest levels of the rate ranges (2 lb a.i./A for < 20 inches rainfall; 4 lb a.i./A for > 20 inches rainfall) would only be used in restricted areas on rangeland to control localized growths of difficult to control brush species. Fourth, the Marietta, Oklahoma study appears to be an outlier when compared to the three other residue monitoring studies (MRID's 406400-01, 406400-02, 406400-04). In these studies at application rates of 1 to 3 lbs a.i./acre, tebuthiuron concentrations in catchment ponds were 0.002 to 0.07 ppm. (See Table 2)

In determining a reasonable worst-case exposure concentration for tebuthiuron, DowElanco used a "Back of the Envelope" calculation. We assumed that tebuthiuron was applied to a 10-acre watershed at a maximum labeled use rate on rangeland of 4 lb a.i./A. If 5% of the amount of tebuthiuron applied runs off the watershed into a 1 acre

pond that is 6 feet deep, the catchment pond would contain 0.123 ppm of tebuthiuron.

Evaluation for Non-Target Aquatic Plants - Response to EPA Review

DowElanco tries to follow methods that are accepted by scientists and regulatory bodies around the world. EPA standards normally provide explicit guidelines or references for the conduct of studies. The EPA Standard Evaluation Procedure for Non-Target Plants (1986) and Pesticide Assessment Guidelines, Subdivision J (1982) do not, however, provide detailed guidance for analysis of results from studies with aquatic plants. They omit any recommendation for the parameter which should be used to calculate an EC50 value. DowElanco, therefore, referred to the Office of Toxic Substances Final Rule (1989), U.S. EPA Algal Assay Bottle Test (1971), and OECD Guidelines (1984) and calculated EC_{50} values for phytotoxicity based on growth rate.

It appears that the contractor reviewing the tebuthiuron studies for the EPA calculated EC_{50} values for various time points based on standing crop, not growth rate, and then generally selected the lowest EC_{50} value. We do not believe this is appropriate, as the EC_{50} values based on standing crop constantly change with time. An article by Niels Nyholm (Water Research-1985, Vol. 19, No. 3, pp 273-279) discusses the pros and cons of using standing crop and growth rate as response variables in algal toxicity tests. Nylom concluded that for systems with predominantly exponential growth, growth rate is superior to standing crop. He also stated that results from interlaboratory comparison tests varied less when based on growth rate than when based on standing crop. A copy of Nyholm's paper is attached for your reference

Since the rate of increase of an aquatic plant population is logarithmic in guideline studies, the ecological success of the population is better measured by growth rate and not standing crop. EC_{50} values based on standing crop vary with time and are affected by the magnitude of the specific growth rate. As an example, in a 14-day algal toxicity test with tebuthiuron and Selenastrum capricornutum (Accession No. 252491), the maximum specific growth rate of control populations led to nutrient depletion and stable algal populations after about 5 days. Prior to day 5, algal populations were in the logarithmic phase of growth. If we look at the standing

crop of control algae on day 3 during this phases of growth, we find the standing crop to be 1.1 X 10^6 cells/ml. The EC_{50} for day 3 based on standing crop was 33.9 ug/L. Through interpolation of the dose-response curve, the level of algae present at this EC_{50} concentration would be approximately 0.55 X 10^6 cells/ml. The standing crop at this EC_{50} concentration (33 ug/L), which was an actual test concentration in the study, increased in the next 24 hours more than 3 fold to approximately 2.2 X 10^6 cells/ml. So the standing crop at the calculated EC_{50} increased from day 3 to a level (2.2 X 10^6 cells/ml) on day 4 that was 2 times higher than the standing crop of controls on day 3. This time lag of less than on day is probably insignificant, ecologically. However, if we look at the EC_{50} based on growth rate, we saw quite a different trend. In this study, the EC_{50} based on maximum specific growth rate was 305 ug/L. The standing crop equivalent to this concentration on day 3 was about 0.04 X 10^6 cells/ml. Based on growth rate it would take between 7 and 11 more days for the standing crop to reach levels similar to the control level on day 3. This time lag is obviously significant, ecologically.

The contractor also recalculated EC_{50} values for growth rate inhibitions using probit analysis. The reviewer obtained EC_{50} values of 15.1 ppm for Anabaena, 0.193 ppm for Navicula, and 0.102 ppm for Skeletonema. Except for the EC_{50} value for Anabaena, these values are not much different than those calculated by DowElanco. We would like to mention, however, that expressing percent inhibition of specific growth rate, a non-discrete variable, as a probit is probably inappropriate in order to calculate an EC_{50} value. Probit analysis is appropriate for the analysis of sample proportions obtained from a binomial sampling scheme (i.e., quantal response data such as mortality), not for a non-discrete sampling scheme (i.e., growth rate). Furthermore, the probit transformation is not defined at negative values which are possible when calculating inhibition of growth rate. Statistical literature discusses the use of probit analysis which supports this argument. One commonly referenced source is D.J. Finney's 1971 book, entitled Statistical Method in Biological Assay. Throughout the text, Finney indicates that probit analysis is used appropriately when analyzing quantal response data.

As can be seen from Table 3, there is a considerable difference in the EC_{50} values calculated in our reports and those calculated by the contract firm reviewing our data. Based on growth rates, the EC_{50} values for our studies were 30.9 ppm for Anabaena, 0.213 ppm for Navicula, 0.101 ppm for Skeletonema, and 0.305 ppm for Selenastrum. We feel that these EC_{50} values accurately represent the results of our studies and should be used for any risk assessment evaluation.

Issue of Test Procedures - Response to EPA Review

1. The use of aluminum foil on top of flasks to "prevent contamination while allowing free gas exchange" was questioned for the toxicity studies of tebuthiuron to Anabanena, Skeletonema, and Navicula (MRID's 41080401, 41080402, and 41080403) because it may not allow for free gas exchange. We believe that this procedure did not compromise the studies in that cell counts of the control populations were approximately 2.5 to 5.0 million cells/ml, indicating healthy algal populations and an adequate gas exchange in the test vessels. Further, according to the U.S. EPA Algal Assay Procedure Bottle Test (1971) loose fitting aluminum foil can be used as a flask enclosure while still allowing free exchange of gas.

2. In the toxicity study of tebuthiuron to Lemna (MRID 41080404) only three plants per replicate were used. The SEP recommends use of five plants per replicate for Lemna. The use of three plants in this research program was reviewed and agreed to by Mr. Charles Lewis of the Ecological Effects Branch in conversation and correspondence with Mr. Patrick Cocke of Lilly Research Laboratories prior to the start of the study. Based on growth rate analysis, the EC_{50} for Lemna was 0.235 ppm in this study.

With this more comprehensive explanation of how tebuthiuron is used, it is trusted that a critical and realistic re-evaluation of the environmental risks that first led to the request for Tier III aquatic and terrestrial studies will be made by the Ecological Effects Branch. In the case of terrestrial risks, tebuthiuron applications are not likely to be off-target. Spray applications

are, as with other liquid treatments, directed to the target site through ground operated spray equipment. Further, the vapor pressure of tebuthiuron is very low (1 X 10^{-7} mm Hg at 25°C) plus it is not active except as a soil treatment and as it is taken up by plant roots. In the case of granular applications, treatments again are with ground operated spreaders and treatment is directed at the treated site. The carrier is an "8/16 mesh" limestone product with a bulk density of 78-88 lb/cu. ft. These heavy particles are influenced very little by ground breezes. Pellet applications are influenced even less by ground breezes which help assure that even with aerial applications, as with broadcast treatments to rangeland, treatment patterns remain distinct.

In the case of aquatic risks from use of tebuthiuron, even the realistic worst - case exposure scenario does not appear to pose a significant threat to aquatic plant populations. If EC_{50} values based on growth rate (Table 3) are compared to the EEC (0.123 ppm) derived from a realistic worst-case calculation, only the marine diatom (Skeletonema) EC_{50} is below the EEC. Due to the volume of the receiving water in a marine environment, Skeletonema would likely never be exposed to the highest expected tebuthiuron concentration calculated for a pond.

This information is provided with our sincere intent to accurately represent the use of tebuthiuron under current use and commercial conditions. If additional detail of any of the information presented or other dimensions on the use of tebuthiuron are desired, please contact me at my new telephone number (317) 870-7266. I will be more than anxious to work with you in any way you ask.

Sincerely,

DOWELANCO

Merlyn L. Jones, Ph.D.
Product Registration Manager

MLJ/af

Tebuthiuron

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Pages _____ through _____ are not included.

The material not included contains the following type of information:

____ Identity of product inert ingredients.

____ Identity of product impurities.

____ Description of the product manufacturing process.

____ Description of quality control procedures.

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____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

TABLE 2. Characteristics of Field Residue Monitoring Sites in Studies With Tebuthiuron and Highest Observed Concentration (PPM) in Catchment Pond.

State	Texas	Idaho	Oklahoma	Arizona
Area of Applied Watershed (A)	28.9	98	11	168
Size of Catchment Pond (Acre Ft.)	0.1	N/A	1.5	N/A
Rainfall (In/Yr)	16	52	44+ (7" in 1 day)	N/A
Application Rate (lbs/Acre)	2	1	2	3
Highest Observed Tebuthiuron Conc. (ppm) in Catchment Pond	0.07	0.002	0.18	0.05

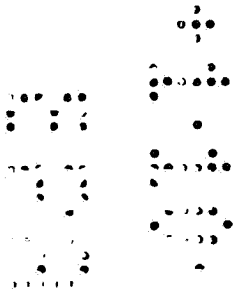

Table 3. Comparison of EC50 Values Calculated by DowElanco and by an EPA Contractor for Several Studies with Aquatic Plants Exposed to Tebuthiuron.

Test Species	DowElanco EC50 Values (ppm) for Tebuthiuron Based on Growth Rates		EPA Contractor EC50 Values (ppm) for Tebuthiuron Based on Standing Crop
		Mean Area	
Duckweed	0.235	0.16	0.135 (0.234)[a]
Green Alga	0.305	0.05	0.0496
Blue-Green Alga	30.9	2.18	4.06 (15.1)[a]
Freshwater Diatom	0.213	0.07	0.081 (0.193)[a]
Marine Diatom	0.101	0.047	0.067 (0.102)[a]

[a]Based on probit analysis of growth rate by EPA contractor.

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REVIEW PAPER

RESPONSE VARIABLE IN ALGAL GROWTH INHIBITION TESTS—BIOMASS OR GROWTH RATE?

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(*Received July* 1983)

Abstract—A number of response variables can be derived from growth inhibition toxicity experiments with microorganisms: currently, there is a controversy regarding which variable to specify in standardized test protocols with algae. Yield or biomass at a specified time is the variable used most frequently. Specific average growth rate is a debated alternative, and appreciable differences may exist between EC figures derived from the same set of data using either test endpoint.

The problem is reviewed and analysed mathematically. It is concluded that from a theoretical point of view, growth rate is a better response variable than biomass, e.g. because EC figures estimated from growth rates are less dependent on particular test system parameters. However, as the use of both methods to analyse data will probably continue, it is important to be aware of the potential differences between estimated EC figures.

Key words—toxicity, growth inhibition, algae, response variable, endpoint, test-protocols, standardization, growth rate, biomass

INTRODUCTION

Recent attempts to standardize growth inhibition tests with microalgae (referred to in the following as "algal toxicity tests") for regulatory purposes, have revealed a number of methodological problems. Interlaboratory comparison programmes carried out under the auspices of, for example the International Standards Organization (ISO) (Hanstveit, 1980) and the Nordic Council of Applied Research (Källqvist *et al.*, 1980) have demonstrated that most typical test procedures hitherto in common use were not comparable.

With identical algal test species, EC_{50} estimates could differ between laboratories by more than a factor of 1000, even for simple chemicals.

The lack of comparability between different methods seems primarily to be due to a number of physical/chemical factors which either differed between methods or which were controlled inadequately (see, for example, Nyholm, 1982; Källqvist, 1982; Nyholm and Källqvist, 1984). It has been realized, however, that also the method used to analyse the data and to express the test endpoint may significantly influence the derived EC figures (including the EC_{50}) that are stated as the results of a test.

At present there is an unresolved controversy, essentially between two different schools regarding selection of response variable in algal toxicity tests. One advocates the use of biomass (or some figure related to biomass), and the other some measure of specific growth rate. The percentage of inhibition is quantified in both cases by relating to the response in control cultures with no toxicant added. The topic is currently being debated heavily within expert committees of the ISO and the OECD (Organization for Economic Cooperation and Development) engaged in the development of algal toxicity test protocols.

Until now, no agreement on calculation method has been reached, however, and finalized and approved protocols will probably include both options and leave the choice to the individual experimenter.

GENERAL DISCUSSION

Basic test design principles

During the past decade, algal assays have been widely used as growth potential tests to assess the nutritional status of natural waters (e.g. Skulberg, 1966; U.S. EPA, 1971). The endpoint in these types of assays is usually the ultimate algal biomass or yield as reached, e.g. after 14 days. Although this final yield may be influenced by the presence of toxicants, for most applications it does not provide a good endpoint for toxicity tests. The reasons include: (1) even considerable reductions in growth rate may not result in changes or may be associated with only minor changes in final yield, because the toxicant-affected cultures may gradually "catch up" with the controls when nutrients become limiting; (2) in the course of the test, toxicity can be lost due to various mechanisms and thus cause little or no effect on the final yield (see, for example Walsh *et al.*, 1982).

Thus, it appears to be generally agreed that for most purposes algal toxicity tests should be of rela-

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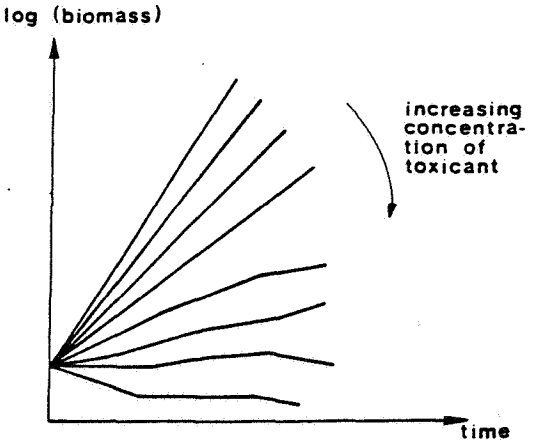

Fig. Constructed example of growth curves obtained from an algal toxicity experiment. Control cultures and slightly to moderately inhibited cultures are supposed to grow exponentially, while severely inhibited cultures grow irregularly.

tively short duration and terminated well before the growth of the control cultures becomes severely depressed because of limiting factors.

Accordingly, the current position of the ISO and the OECD is that tests should be designed so that control cultures grow exponentially for the whole duration of the test. The performance of an algal toxicity test with exponential growth is illustrated in principle in Fig. 1. It should be noted that although the growth pattern is predominantly exponential, irregular growth may occur in inhibited, and in particular in severely inhibited, cultures.

The controversy

The ongoing debate on choice of response variable in algal tests is not only a question about the numerical figures reported from a toxicity experiment. It also deals with the scientific rationale behind testing and with the intended use of the results. Additionally, there are certain problems of purely practical nature to be considered. Arguments put forward in favour of biomass (e.g. as measured at the end of the test or at the time where exponential growth in control cultures ceases) are, for example, (1) simplicity, (2) "direct interpretation without any assumptions necessary on the mode of growth", (3) the circumstance that generally lower and thus more "sensitive" EC_{50} values are obtained and (4) toxic effects are detected more easily since small changes in growth rate result in much larger changes in biomass.

Conversely, it can be argued that what makes sense from an ecological viewpoint is how the growth rate is affected, because the growth rate is decisive in determining the competitive success of an algal species in a dynamic natural ecosystem. Disregarding any possible ecological interpretation, it has been argued from a theoretical viewpoint that specific growth rate should be preferred over biomass simply as a consequence of the very nature of the exponential mode of growth in the test system. Thus, because of the exponential growth pattern, EC figures derived from biomass vary with time and are also affected by the absolute magnitude of the specific growth rate, i.e. they are test system specific. Therefore, it can be anticipated that using some growth rate estimate as response variable will provide greater reproducibility and comparability of test results between laboratories, than if biomass is used.

Further, it makes no logical sense to claim that biomass provides a more "sensitive" estimate than growth rate. The fact that EC_{50} figures calculated from biomass are usually numerically smaller than corresponding figures calculated from growth rate, is simply a mathematical consequence of the exponential growth pattern. Finally, biomass does not reflect toxic effects more sensitively than, for example, average growth rate because the numerical differences between the results are larger; so is the variance of the data points.

Arguments to the contrary from supporters of biomass include that a prerequisite for using growth rate and for the theoretical arguments is strict exponential growth, which, as already pointed out, not necessarily takes place in toxicant affected cultures, so therefore using growth rate is no solution. The time and system specific dependency of EC figures estimated from biomass can be estimated to a sufficient extent by strict standardization of test conditions and by specifying an exact time for the biomass endpoint measurement, e.g. 72 h after inoculation.

The other camp may then argue that one should not "over-standardize" if this is not necessary, and as to the problem of deviations from exponential growth in inhibited cultures: yes, the problem has been acknowledged, but first, the lower part of the concentration effect curve, e.g. from 0 to 50% inhibition, represents the part which is interesting in ecotoxicology, and slightly inhibited cultures normally grow exponentially or close to exponentially. Second, although growth in inhibited cultures may be irregular, the growth pattern—if appreciable growth occurs at all—is usually much closer to an exponential pattern than to a linear pattern. In fact, it can be claimed with equal justification that an assumption inherent in the prescription of biomass as response variable is that growth must be linear. Therefore, concern about deviations from strict exponential growth in inhibited cultures if growth rate is used seems to be a bit out of place.

In needs mentioning, too, that another aspect of the controversy is fundamentally different attitudes towards the need for precision and reproducibility in toxicity testing in general. Some hold the position that tests need not be very reproducible nor strictly standardized since in an intended context of a preliminary hazard evaluation of chemicals, e.g. for notification purposes, a precision within an order of

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magnitude is claimed sufficient. Another point of view is, of course, that toxicity tests have a much wider potential use, e.g. in testing of effluents for regulatory purposes, in environmental impact studies or for defining water quality criteria. While for such purposes site specific variables, such as the type of water, should normally be considered, it still makes sense to standardize certain general test principles. In algal tests, e.g. a number of validity criteria and the method used for treatment of data.

Irrespective of the intended purpose of testing it may, however, simply be argued that what are the objections against attempts to increase test reproducibility, if the costs of testing are not increased, or are increased only insignificantly?

The choice of response variable in algal toxicity tests is one such factor that by standardization could improve the comparability of test results with only marginal consequences for the costs.

Having attempted to present an outline of the various aspects of the on-going debate regarding selection of response variables in algal toxicity tests, the purpose of the rest of the paper is briefly to review the state of the art from a technical scientific point of view and to present a mathematical analysis of the problem.

The topic has been dealt with in a number of ISO documents (including also a mathematical treatment by Rhône-Poulenc, 1982) and in technical reports of limited circulation, but very little has been published in the open literature.

STATE OF THE ART

Inhibitory effects in algal growth tests may be expressed in several ways (for example refer to Blankley, 1973) and other endpoints than biomass or growth rate can be identified. By far the most common procedure for evaluating results, however, has been to use the relative biomass as recorded at the end of the test, i.e.

$$\%\text{ inhibition} = \left[1 - \frac{X'(T)}{X(T)}\right] \cdot 100 \qquad (1)$$

where $X'(T)$ and $X(T)$ represent the biomass in test cultures and in control cultures at time T which is the test duration. Ideally, biomass is expressed in terms of dry weight, but usually some surrogate meausre is used, such as cell volume or cell number as determined with electronic particle counters, optical density, fluorescence or chlorophyll concentration.

It is normally accepted in routine testing that the ratio of the measured variable to biomass may change somewhat from control cultures to inhibited cultures without affecting the results much. Examples from the literature on the use of biomass as response variable are numerous. Test protocols for routine testing which prescribe this method include Miller *et al.* (1978), Joubert (1980), ISO (1982) and U.S. EPA (1982).

Some measure of growth rate as the response variable has been used less often. Examples of applications in routine bioassay include Källqvist (1978), Payne and Hall (1978), OECD (1981), Damgaard and Nyholm (1982) and Walsh *et al.* (1982). The simplest growth rate estimate is probably the "average growth rate" μ_{av}, (Källqvist, 1978, 1982; OECD, 1981) calculated as:

$$\mu_{av} = \frac{\ln X(T) - \ln X_0}{T} \qquad (2)$$

where X_0 is the inoculated biomass concentration (at time zero). Due to technical difficulties associated with measurements at low cell densities X_0 is normally determined most accurately as a nominal concentration (cell density in the pre-culture used for inoculum divided by the dilution factor) rather than as a directly measured concentration.

Alternatively, the growth rate may be estimated from a plot of log-transformed biomass measurements vs time, e.g. by linear regression. (It needs mentioning here that the variance of the biomass measurements at low cell densities may be quite large after log-transformation which may necessitate a proper statistical weighting procedure, or alternatively demand that an exponential function is fitted directly to untransformed data using a non-linear regression technique.)

If a lag phase occurs, some may prefer to use only the linear part of the growth curve to estimate the maximum growth rate while others take the average growth rate over the whole test duration. The current attitude within the ISO and the OECD is, however, that lag phases in the control cultures should be avoided by inoculating with exponentially growing cells propagated under the test conditions. Still, a toxicant induced lag phase is possible, and this type of toxic effect may be ignored, if only data points representing the subsequent linear curve portion are included in the analyses. This does not necessarily have much influence on the overall results, but can be taken into account by using the concept of average growth rate [e.g. as calculated from equation (2)], rather than a regression estimate of the linear curve portion.

A recent proposition (Nusch, 1982, 1983) which has been adopted by both the ISO (1983) and the OECD (1983) is to use the area under the growth curve ("area comparison method"). The area is calculated most easily by trapezoidal integration which is equivalent to constructing the growth curve by connecting the data points with straight lines. The idea behind this procedure is to extract as much information as possible from the data available without relying on a model nor on any assumption on the growth pattern.

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The current proposition is to use directly measured biomass values without log-transformation, i.e. the growth curve is plotted on a linear scale. For a test system with exponential growth this implies, however, that the area comparison method, in principle, is related to biomass based methods, although more elaborate. If log-transformed biomass measurements are used on the other hand, the method can be regarded simply as another way of estimating an average growth rate.

Therefore, although the idea of using the area under the growth curve (which is easily calculated by a computerized procedure) deserves attention, the basic issue of the controversy on response variable in algal toxicity tests remains: biomass or growth rate?

Practical experience on differences between biomass derived EC_{50} estimates, termed EC_{x50} (Hanstveit, 1982) and EC_{50} estimates derived from growth rates, termed EC_{r50}, has been obtained from two subsequent ring-tests of a proposed ISO draft standard method with freshwater green algae (*Selenastrum capricornutum* and *Scenedesmus subspicatus*). The results were compiled by Hanstveit and Oldersma (1981) and by Hanstveit (1982), who analysed the raw data as submitted by the participating laboratories using both a biomass based method and a method based upon average growth rate. From the first ring-test (test substances: $KClO_3$, $K_2Cr_2O_7$, 3,5-dichlorophenol and $CuSO_4$) was concluded that EC_{x50}'s were generally lowest and could differ by a factor of at least 2 from EC_{r50}'s. The next ring-test (test substances: $K_2Cr_2O_7$ and 3,5-dichlorophenol) revealed that EC_{x50}'s showed a higher degree of scatter among laboratories than ED_{r50}'s, and again EC_{x50}'s were about a factor of 2 lower than EC_{r50}'s.

Analysing the same data Nusch (1983) found no significant differences between EC_{50} values calculated from area under growth curve (untransformed data) and from test end biomass, respectively. Likewise, EC_{50} values calculated by means of the area comparison method using log-transformed data were not significantly different from corresponding figures calculated from average growth rates. A problem of great uncertainties associated with log-transformed biomass numbers at low cell densities was identified and used as an argument for not recommending log-transformation. As pointed out previously, however, the alteration of the variance distribution caused by log-transformation may demand a more complicated calculation procedure. Alternatively, biomass measurements near the limit of detection should simply not be used, and the nominal inoculated biomass concentration be taken as the starting point.

Compiling results from an interlaboratory comparison study within the Nordic countries, Källqvist (1980, 1982) demonstrated that dose–response curves as recalculated from the raw data submitted by 3 laboratories using relative growth rates as response variable, could be superimposed almost exactly, although the EC figures as reported by the laborat differed somewhat. The three laboratories all usec green alga *Selenastrum capricornutum* and the growth medium. Test conditions differed, howe with respect to such factors as temperature, intensity, mass transfer conditions for CO_2, and duration. The chemicals tested were: Anis sodium-laurylsulphate and phenol.

While in the ISO ring-tests EC_{x50} and EC_{r50} diff by a factor of about 2, potentially however, difference can be considerably larger for other ch icals and other test systems. This is shown be through a mathematical analysis of the probl Practical examples that support this statement clude Blanck (1983) who calculated EC_{x50}, EC_{r50} r for 6 chemicals tested against 4 different algal spec and who found a range as large as 0.19–8.1. author (unpublished data) has experien differences between EC_{x50} and EC_{r50} up to abou factor of 5 using ISO (1982) or U.S. EPA (Mille *al.*, 1978) standard test methodology.

MATHEMATICAL ANALYSIS

The following analysis is confined to tests wh the basic growth pattern is approximately ponential throughout the test duration. It is furt presupposed that the cultures are exposed to c tinuous light and therefore are growing at a const rate. Continuous light is prescribed in most protocols for practical reasons.

Assuming that strict exponential growth prev the growth of a control culture can be described the equation:

$$X(t) = X(t_0) \cdot \exp[(\mu_m)(t - t_0)]$$

where $X(t)$ and $X(t_0)$ is biomass (dry weight) conc tration at time t and t_0 respectively, t_0 is the lag ph duration (see below) and μ_m is the maximum spec growth rate characteristic of non-limited, n toxicant-affected growth in the particular test syste Let us, for reasons of simplicity, further assume th over a certain range of concentrations of test ma rial, C, the specific growth rate, μ, changes linea with the logarithm of the concentration, z. Thus

$$\mu(\zeta) = \mu_m(1 - \alpha\zeta).$$

The dose metameter ζ in equation (4) is defined

$$\zeta = z - z_0 = \log C - \log C_0$$

where z_0 is the extrapolated abscissa corresponding no effect [$\mu(\zeta = 0)/\mu_m = 1$] and C_0 the equivale concentration. The justification for the assumption approximate dose–response linearity over a certa range is general practical experience from bioass For a similar treatment see Finney (1978).

Now, using the relative specific growth ra $Y_r = \mu(\zeta)/\mu_m$ as the response variable, the line dose–response curve segment we confine ourselves

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is given by

$$Y_r(\zeta) = 1 - \alpha\zeta. \quad (6)$$

The equation describing the growth of biomass, X', in a culture exposed to test material in a concentration within the above range of dose response linearity then becomes:

$$X'(t, \zeta) = X'(t_1) \cdot \exp[(\mu_m)(1 - \alpha\zeta)(t - t_1)] \quad (7)$$

where t_1 is the lag phase duration. Thus, the relative biomass $Y_b = X'/X$ can now be calculated combining equation (3) and (7)

$$Y_b(t, \zeta) = \frac{X'(t_1)}{X(t_0)} \cdot \exp[(-\mu_m) \times [\alpha \cdot \zeta(t - t_1) - (t_0 - t_1)]] \quad (8)$$

with a dose-dependent slope, β, equal to

$$\frac{\partial Y_b}{\partial \zeta} = \beta = -\mu_m \cdot \alpha \cdot (t - t_1) \cdot Y_b(t, \zeta). \quad (9)$$

If no lag phase occurs, and if the cultures are inoculated to exactly the same cell density, equations (8) and (9) simplify to

$$Y_b(t, \zeta) = \exp[(-\mu_m) \cdot \alpha \cdot \zeta \cdot t] \quad (8a)$$

$$\frac{\partial Y_b}{\partial \zeta} = \beta = -\mu_m \cdot t \cdot \alpha \cdot \exp[(-\mu_m) \cdot \alpha \cdot \zeta \cdot t]. \quad (9a)$$

Thus, it follows from the assumption of exponential growth that using relative biomass as the response variable results in numerical toxicity estimates and dose–response curve slopes which depend on the test duration, the absolute magnitude of the maximum specific growth rate μ_m (and thus particular system parameters), and which are also influenced by differences in inoculated cell concentrations and by the occurrence of lag phases. In contrast, however, and with the same basic assumption of exponential growth, toxicity estimates derived from growth rates do not depend on these factors. Therefore, from a theoretical viewpoint, growth rate can be claimed to be a superior response variable. The theoretical superiority of growth rate relative to biomass is a consequence of the very nature of exponential growth and can be expected to hold true as long as the growth pattern is closer to being exponential than to being linear.

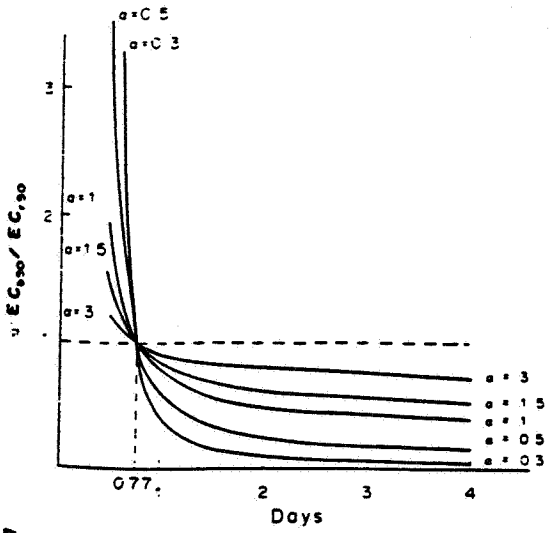

Fig. 2. Plot of the ratio EC_{b50}/EC_{r50} as calculated from a theoretical equation [equation (12a)] assuming a specific growth rate of 1.8 days^{-1}. α is the slope of the dose–response curve for relative growth rate vs log (concentration).

EC_{50} values as derived from the two different response variables come out as follows:

Based upon growth rate:

$$EC_{r50} = 10^{\left(\frac{0.5}{\alpha} + z_0\right)}. \quad (10)$$

Based upon biomass:

$$EC_{b50} = 10^{\left(\frac{\left[\ln \frac{2X'(t_1)}{X(t_0)}\right] + \mu_m(t_0 - t_1)}{\mu_m \cdot \alpha \cdot (t - t_1)} + z_0\right)} \quad (11)$$

or as derived from equation (8a):

$$EC_{b50} = 10^{\left(\frac{\ln 2}{\alpha \cdot \mu_m \cdot t} + z_0\right)}. \quad (11a)$$

Therefore we expect, in general, that EC_{r50} differs from EC_{b50}. Their ratio with no lag phase is

$$EC_{b50}/EC_{r50} = 10^{\left[\frac{1}{\alpha}\left(\frac{\ln 2}{\mu_m \cdot t} - 0.5\right)\right]}. \quad (12a)$$

The ratio approaches infinity as α tends to zero, while for large α's ("threshold" or "all or nothing" types of effects), the ratio approaches unity. For test durations shorter than $t = 2\ln(2)/\mu_m$ (e.g. 0.77 days for $\mu_m = 1.8$ day^{-1}) EC_{b50} exceeds EC_{r50}, while for longer test times EC_{b50} is smaller than EC_{r50} and continues to diminish with time until the ratio reaches the asymtotic value $10^{-0.5\alpha}$.

EC_0 or "no effect" values calculated by either method are of course identical, and at the other end of the dose–response curve, near EC_{100}, EC_b and EC_r estimates approach each other again. At neither extreme end, does dose response linearity prevail, however, for which reason the above simplified mathematical analysis is not valid. It can be concluded, though, by simple considerations, that e.g. the EC_{10}, which in addition to the EC_{50} also is a frequently reported key result from ecotoxicological tests, is less dependent on the choice of response variable than is the EC_{50}.

Figure 2 is a plot of the ratio EC_{b50}/EC_{r50} calculated from equation (12a) and assuming a maximum

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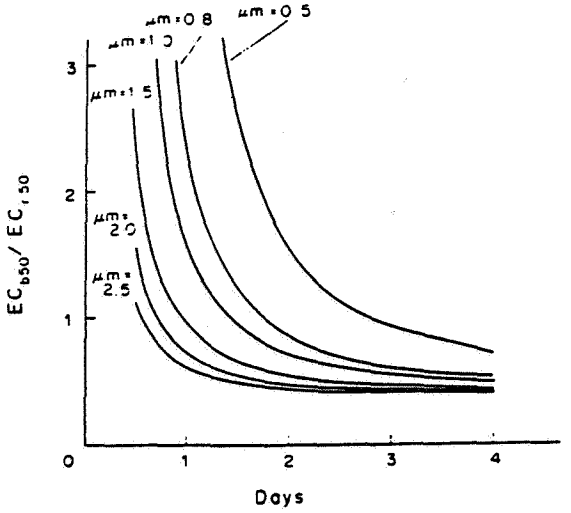

Fig. 3. Plot of the ratio EC_{b50}/EC_{r50} as calculated from a theoretical equation [equation (12a)] for specific growth rates from 0.5 to 2.5 days^{-1} assuming a slope, α, of the dose–response curve for relative growth rate vs log (concentration) equal to 1.

specific growth rate $\mu_m = 1.8$ days^{-1} which is typical for a freshwater green alga (e.g. *Selenastrum capricornutum*) grown under standard test conditions (ISO, 1983; OECD, 1983; Miller *et al.*, 1978) (approx. 24°C, saturating light and continuous shaking). A representative range for the dose–response curve slope α, of 0.3–3 has been selected based on the author's experience. The steepest curve slope ($\alpha = 3$) has been observed for copper (Nyholm, unpublished results) while a slope within an interval of about 0.5–1.5 seems to be more common. References to published dose–response curves of growth rate vs concentration, from which approximate slopes can be deducted, include Källqvist (1978, 1982) and Damgaard and Nyholm (1982).

It is seen from Fig. 2 that the ratio EC_{b50}/EC_{r50} varies considerably at short test durations, but assumes an approximately constant value after 2 days (for the particular growth rate of 1.8 days^{-1}).

Figure 3 is a plot of EC_{b50}/EC_{r50} likewise calculated from equation (12a) and now illustrating the influence of μ_{max} for an intermediate curve slope of $\alpha = 1$. It is seen that short test times and low growth rates lead to a large difference between the two EC_{50} estimates, while again the their ratio tends towards a constant value, if the test time is long enough.

CONCLUSIONS

Practical experiences as well as theoretical considerations reveal that appreciable differences may exist between EC figures obtained from algal toxicity tests depending on whether biomass or growth rate is taken as the test endpoint. For test systems with predominantly exponential growth, theoretically, growth rate is superior to biomass, and practical ring-test excercises have shown that EC_{50} values derived from "average growth rates" showed less scatter among laboratories than EC_{50} values calculated from biomass. However, for a number of reasons disagreement prevails regarding which response variable to prescribe in standardized test protocols.

Users of test results should therefore be aware of the differences between EC figures as calculated using either method, and experimenters should likewise make their choice after having given the problem careful consideration. It is the hope of the author that this paper has contributed not only to drawing attention to the controversy on response variable, but also to elucidate the problem.

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